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The MMTV retrovirus causes mammary adenocarcinomas in mice by proviral insertion near members of the *wnt* family of proto-oncogenes, leading to their deregulation and cellular transformation. The 5' end of the MMTV LTR has been implicated in tissue-specific activation of these genes. In this study, we characterize an enhancer element (Ban2; -1075 to -978) at the 5' end of the MMTV LTR. We show that this enhancer is 5-fold more active in a murine mammary carcinoma cell line (34i) than in a fibroblast cell line (NIH3T3), and is inactive in the liver carcinoma cell line HepG2. Mutagenesis of the enhancer reveals four cis-acting elements that are required for maximal activity. DNA-binding proteins that interact with each of the four elements have been identified. One of these factors, designated mp5, is either identical to, or closely related to, the transcription factor AP-2. The mp5/AP-2 DNA-binding activity co-migrates with recombinant AP-2 and is supershifted by anti-AP-2 antibodies. We also show that the lack of enhancer activity in HepG2 cells results from the absence of AP-2 protein in these cells. Co-transfection of an AP-2 expression vector restores the activity of this enhancer in HepG2 cells.

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Julia Machett, Ph.O. 10-10-95

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Introduction

Mouse mammary tumor virus (MMTV) is a slowly transforming B type retrovirus that infects several different tissues including mammary, lungs, kidney, salivary glands, testes, and lymphoid tissue. Exogenous MMTV infection is milk-borne and the highest viral replication occurs in the mammary gland during lactation. Endogenous MMTV provirus is found incorporated into the genome of many strains of mice and is transmitted vertically through the germline. It is well established that the MMTV provirus is induced by several steroid hormones including glucocorticoids and progestins. This induction is mediated via hormone responsive elements (HREs) in the MMTV promoter (1).

MMTV predominantly causes mammary adenocarcinomas but can also less frequently cause T-cell leukemias, thymomas, and kidney adenocarcinomas in mice (2-5). It is of great interest to understand why MMTV primarily causes mammary adenocarcinomas even though the virus infects several tissue types. In mammary tumors MMTV has been found to be integrated near several endogenous proto-oncogenes called *wnt* genes (6). The *wnt* genes are developmental genes, normally expressed only during embryogenesis, that become deregulated by proviral insertion. It is the aberrant expression of these proto-oncogenes that contributes to neoplastic transformation. *wnt*-2 gene regulation has been shown to be refractory to hormone stimulation; the locus is constitutively expressed in MMTV-induced mammary tumors, indicating that activation of *wnt*-2 is due to long terminal repeat (LTR) elements independent of the hormone response elements (HREs) (7). The fact that MMTV predominantly causes mammary adenocarcinomas suggests that retroviral activation of the *wnt* genes is driven by mammary-specific transcriptional elements of the provirus. In addition, viral integration upstream of the *wnt* genes almost always occurs in the opposite transcriptional orientation relative to the activated gene, suggesting that the MMTV promoter must be positioned correctly

for deregulated expression of the *wnt* genes (7,8).

The contribution of the 5' end of the LTR to the tissue specificity of MMTV expression has been studied in transgenic mouse models and in cultured cells. Stewart and colleagues reported that a growth hormone reporter gene driven by an LTR with a deletion between -863 and -110 had a restricted tissue distribution, similar to a construct driven by the full length 1320 bp LTR (9). This study suggested that sequences from -1185 to -863 along with the MMTV minimal promoter were sufficient for tissue specific expression of the LTR. In a later study, Ross and colleagues showed that a slightly smaller fragment of the LTR (-1160 to -987) can direct expression of a heterologous promoter to the mammary gland in transgenic mice (10). Our previous work also identified a sequence at the 5' end of the MMTV LTR (-1180 to -968) that has enhancer activity specific to cultured mammary cells (11). A factor binding to this region, designated mp4, was detected by electrophoretic mobility shift assays (EMSAs), and was shown to generate a DNaseI footprint between -1078 and -1052. Cato and colleagues reported that a larger DNA fragment (-1094 to -739) directed mammary-specific activity in cultured cells, and showed that several proteins bound to this region by *in vitro* DNaseI footprinting (12). Ishimoto and colleagues have also localized a mammary specific activity to a 5' fragment of the LTR flanked by two BanII restriction enzyme sites (-1075 to -978), designated Ban2 (13).

In this study, we show that when assayed in DNA transfection experiments, the Ban2 enhancer cloned upstream of a heterologous promoter stimulates basal transcription to a level 5-fold greater in mammary cells (34i) than in fibroblasts (NIH3T3) and is inactive in liver carcinoma cells (HepG2). We have characterized this enhancer in mammary cells and non-mammary cells by using site-directed mutagenesis to determine which sequences are required for transcriptional activity. Four elements have been identified as contributing to the activity of this enhancer.

One element, termed mp5, contains a consensus AP-2 DNA-binding site (5'GCCNNNGGC3') (14). AP-2 was originally identified as a sequence-specific DNA-binding protein required for the function of several viral and cellular enhancer elements. There are functional AP-2 binding sites in the enhancers of the SV40, HTLV-1 viruses and the metallothionein IIa, proenkephalin, keratin K14, and murine histocompatibility complex H-2κ^b genes (14,15).

Our results indicate that the mp5 DNA-binding protein is AP-2 or an AP-2 family member. We show that *in vitro* translated, recombinant human AP-2 binds specifically to the mp5 recognition site, and that the AP-2-specific DNA-protein complex co-migrates with the mp5 activity found in mammary cell extracts. We also show that two antibodies to different regions of human AP-2 can supershift the mp5 protein in both human and murine extracts. In addition, we co-transfect a Ban2 reporter plasmid and a eukaryotic expression vector encoding AP-2 into a cell line (HepG2) that lacks endogenous AP-2 and show that co-expression of AP-2 stimulates the otherwise silent Ban2 enhancer. mp5/AP-2 is not the only protein responsible for activation of this enhancer. Functional mutagenesis studies show that three other factors, mp4, F3/NF1 and F12, are necessary for optimal expression of this enhancer. Taken together, our results suggest that the Ban2 enhancer is regulated by a multifactor complex containing at least AP-2, mp4, F3/NF1 and F12.

Materials and Methods

Plasmid Constructs and In vitro Translation

The wild type pBan2TK81 plasmid was constructed by cloning a PCR product (C3H LTR sequence from -1078 to -978) containing HindIII and KpnI restriction enzyme sites into a pTK81 vector digested with HindIII and KpnI. pTK81 contains TK promoter sequences from -81 to +52, relative to the start site of transcription driving the luciferase reporter gene (16). Mutations within the Ban2 fragment designated mp4m, F3m, mp5m, F12m, mp4+5m and (4x)m were all synthesized as single-stranded 127 bp oligonucleotides which were annealed, cut with HindIII and KpnI and ligated into the pTK81 vector cut with HindIII and KpnI. The sequences of these constructs are identical to the wild type pBan2TK81 plasmid except for the mutational changes diagrammed in Figure 3A. All plasmid constructs were confirmed by DNA sequencing.

The AP-2 *in vitro* translation (IVT) expression vector, pT7βSalAP-2, contains the HeLa AP-2 cDNA cloned downstream of the T7 promoter (14). The AP-2 protein was translated from the circular pT7βSalAP-2 vector using the TnT reticulocyte lysate IVT kit (Promega). The AP-2 eukaryotic expression vector, pRSVAP-2, contains the HeLa AP-2 cDNA cloned downstream of the RSV promoter and the control vector contains only the RSV promoter. pBCAT contains a minimal E1b TATA box driving the chloramphenicol acetyl transferase gene and pAP-2BCAT contains 3 AP-2 consensus sites from the human metallothionein IIa gene enhancer (-188 to -159) cloned directly upstream of the E1b TATA box. The cloning of pRSVAP-2, pBCAT and pAP-2BCAT have been described previously (14). The pAP1TK81 plasmid contains 3 AP1 sites from the osteocalcin promoter cloned directly upstream of the TK81 promoter and was kindly provided by Dr. Catherine L. Smith.

Cell Culture and DNA Transfections

All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin at 37°C and 5% CO_2. The 34i cell line was isolated from a mammary tumor induced in a C3H mouse by the C3H-S strain of MMTV (17). NIH-3T3-L1 cells are mouse embryo fibroblasts. T47D and MCF7 cells are derived from a human breast ductal carcinoma and an adenocarcinoma, respectively. HeLa and HepG2 cells are derived from a human cervical carcinoma and a liver carcinoma, respectively. All cell lines except 34i were obtained from the American Type Culture Collection (ATCC).

Transient transfection assays were performed as previously described using the calcium phosphate technique (11). All cells were plated at 1.5-2.5x10^5 in six-well dishes one day prior to transfection. In 34i and 3T3 cells, 1 μg of each reporter plasmid was co-transfected with 50 ng of a plasmid encoding SV40-β-galactosidase (pCH110, Pharmacia). The pCH110 plasmid served as an internal control for transfection efficiency. In HepG2 cells, 1μg of each reporter was co-transfected with or without 50 ng of pRSVAP-2. Luciferase activity was measured using a Berthold LB9500C Luminometer as previously described (11). β-galactosidase activity was measured using a Hoefer TKO 100 Fluorometer using manufacturer recommended protocols. In brief, 5-10 μl of extract was incubated in a final volume of 200 μl containing 25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 2 mM $MgCl_2$, 12 mM β-mercaptoethanol, and .3 mM 4-Methylumbelliferyl-β-D-Galactopyranoside for 30 min at 37 °C. The reaction was stopped with 25 μl of 25% TCA. 50-100 μl was diluted in 2 ml of glycine carbonate (pH 7.8) and β-galactosidase activity was measured using 4-methylumbelliferone as a standard. Results are reported as a ratio of luciferase/β-galactosidase activity. In HepG2 cells where the pRSVAP-2 expression vector was used (Figure 8), the luciferase activity was corrected for by protein concentration because of competing AP-2 sites in the SV40 promoter of the

pCH110 plasmid.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from all cell lines by the method of Dignam et al (18). The buffers were supplemented with protease inhibitors (1 μM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) and 0.1 mM each of benzamidine, leupeptin, pepstatin and aprotinin). The protein concentration of extracts ranged from 2 to 5 mg/ml.

For electrophoretic mobility shift assays, 10 μg of extract was incubated for 30 min on ice in gel shift buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 3 mM $MgCl_2$, 3 mM EDTA, 1 mM DTT, 4% glycerol) and 1 μg poly dIdC with 40,000 cpm of ^{32}P end-labelled double-stranded probe in a 10-20 μl volume. In the assays which contained IVT-AP-2 protein, 1 μl of IVT extract from a typical 50 μl TNT reaction was used. Mobility shift probes were phosphorylated as single-stranded oligonucleotides with T4 polynucleotide kinase in the presence of gamma-^{32}P-ATP (>5000 Ci/mmol), complementary strands were annealed and the double-stranded probe was gel purified. The amount of ^{32}P probe ranged from 5 to 40 fmol per binding assay. All competitors were double stranded and used at 100-fold excess molar concentration (100x) to that of the labeled probe.

For AP-2 supershift assays, 10 μg of cell extract or 1 μl of IVT-AP-2 was preincubated with 1.5 μl antibody in TBST (10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween 20) in an 8 μl volume for 60 min on ice. The protein-Ab solution was then incubated on ice for 10 min with 10 μl 2x gel shift buffer and 1 μg dIdC in a final volume of 19 μl. Finally, 40,000 cpm labelled probe (1 μl) was added and the mixture was incubated on ice for an additional 30 min.

DNA-protein complexes were resolved on a 4% non-denaturing polyacrylamide gel run at 250V at 4°C in 40 mM Tris-HCl (pH 7.4)-5 mM sodium acetate-1mM EDTA. The gels were dried and exposed to a phosphorimager screen

which was analyzed with a Molecular Dynamics Phosphorimager.

Oligonucleotides for Electrophoretic Mobility Shift Assays

All oligonucleotides were annealed with a complimentary oligonucleotide and used in double stranded form. The sequences below represent the coding strand.

mp5 (MMTV, -1044 to -1004)
5' TCG ACC AAA CTT GGC ATA GCT CTG CTT TGC CTG GGG CTA TTG GGG GGT AC 3'

mp5m (MMTV, -1044 to -1004 with 5 bp mutated)
5' TCG ACC AAA CTT GGC ATA GCT CTG CGA ATT CTG GGG CTA TTG GGG GGT AC 3'

mp4 (MMTV, -1075 to -1055)
5' AGC TTC ATC AGA CAA AGA CAT ATT CAG 3'

mp4m (MMTV, -1075 to -1055 with 6 bp mutated)
5' AGC TTC ATC AGG GCT TAA CAT ATT CAG 3'

F2 (MMTV, -904 to -870)
5' AGC TTC TCC TGA GGC AAG GAC CAC AGC CAA CTT CCT CTT ACG 3'

F3 (MMTV, -1051 to -1019)
5' AGC TTC TGC TGC AAA CTT GGC ATA GCT CTG CTT TGC G 3'

F12 (MMTV, -1010 to -980)
5' AGC TTT TGG GGG AAG TTG CGG TTC GTG CTC GCA GGG G 3'

NF1 (MMTV, -81 to -59)
5' TCT TTT GGA ATT TAT CCA AAT CT 3'

NF1m (MMTV, -81 to -59 with both half-sites mutated)
5' TCT TGC AGA ATT TAT AAG AAT CT 3'

ATF2 (adenovirus E4, -47 to -53)
5' AAT TAA AAT GAC GTA ACG GTC TAA AAA ATG ACG TAA CGG T 3'

Met (hMTIIa, -188 to -159)
5' GAA CTG ACC GCC CGC GGC CCG TGT GCA GAG 3'

Antibodies

Anti-AP-2 antibodies Ab53 and Ab57 are affinity-purified rabbit polyclonal antisera raised to the human AP-2 protein. Ab53 was made to an N-terminal peptide and has been described previously (19). Ab57 was made to a C-terminal peptide, CGGNAKSSDKEEKHR (amino acids 425 to 436), and affinity purified as described for Ab53. The affinity purified anti-glutathione-S-transferase antibody was kindly provided by Dr. Emil F. Michelotti.

Results

A 5' MMTV enhancer is preferentially expressed in mammary cells.

We evaluated the activities of various fragments in the 5' LTR to determine their ability to direct tissue-specific expression of a heterologous promoter. We determined that the Ban2 fragment (-1075 to -978) was sufficient to direct preferential expression in mammary cells. For this experiment, the Ban2 fragment was cloned directly upstream of the Herpes Simplex I thymidine kinase (TK) promoter in the pTK81 plasmid (16). Either pBan2TK81 (Ban2) or pTK81 (Vector) were transfected into murine mammary carcinoma cells (34i), murine fibroblasts (NIH-3T3), or human liver carcinoma cells (HepG2). The data in Figure 1 shows that this enhancer stimulates the TK81 promoter 41-fold in 34i cells and 8-fold in 3T3 cells; the Ban2 enhancer is inactive in HepG2 cells. As a positive control to show that the TK81 promoter is functional in HepG2 cells, we transfected a plasmid construct (pAP1TK81) which contains 3 AP1 sites directly upstream of the TK81 promoter. pAP1TK81 was stimulated 294-fold above the pTK81 vector alone thereby confirming that the TK promoter is capable of being activated in HepG2 cells (data not shown). These results suggest that the Ban2 enhancer is preferentially active in mammary cells.

Identification of an activity binding to the (-1032 to -1005) region

In addition to the previously published DNA-binding proteins that have been shown to interact with the Ban2 enhancer region (11-13), we had detected an activity by *in vitro* DNaseI footprinting that bound between -1032 and -1005 in the human mammary carcinoma cell line T47D (20). We designed an EMSA probe, designated mp5 (-1044 to -1004), which encompassed the DNaseI footprint. Several mutations of this DNA sequence were used in gel mobility shift competitions to determine critical sequences for mp5 binding. Figure 2A shows a diagram of the oligonucleotides that were used for this competition experiment. The EMSA

competition (Figure 2B) shows that the mp5 activity denoted by the open arrow is specific; it is competed by unlabeled wild type mp5 but not the unrelated sequence ATF (Lanes 3 and 13). Oligonucleotides containing mutations in the wild type sequence, mp5m, 633, 635 (Lanes 4,6,7), do not compete for binding of this activity to the mp5 oligonucleotide, suggesting that the sequences mutated in these oligonucleotides are important for mp5 binding. Oligonucleotides wapm, 661, 733, 735, 737 and 739 competed the mp5 shift as efficiently as wild type mp5, indicating that the sequences mutated in the oligonucleotides are outside of the mp5 binding site. This shows that the mp5 DNA-binding activity minimally requires sequences within a 12 bp fragment from -1024 to -1012. A Findpatterns search comparing this sequence with sequences in the Transcription Factor Database showed that this element contains a consensus AP-2 binding site (Figure 2A). AP-2 is expressed in a tissue specific manner and was previously shown by Northern blot analysis to be present in the mammary carcinoma cell line MCF7 (21). We next determined if this element or the previously characterized elements were functional in DNA transfection experiments in both mammary and non-mammary cells.

Mutational Analysis of 4 elements in the Ban2 enhancer.

We synthesized specific DNA mutations in the Ban2 enhancer to analyze which DNA sequences contribute to its activity in 34i and 3T3 cells. The mutations are diagrammed in Figure 3A and include 3 elements which had been previously defined by *in vitro* DNaseI footprinting and mobility shift assay (mp4, F3/NF1 and F12) and mp5, a novel DNA-binding activity (11,12). Each of the Ban2 fragments containing a mutation was cloned into pTK81 and the plasmid constructs were transfected into both 34i and 3T3 cells. The data shown in Figure 3B indicates that the Ban2 element is regulated in mammary cells by the combinatorial activity of at least 3 DNA-binding activities. The mutation of a single binding site is not sufficient to eliminate the activity of this enhancer. However, in 34i cells 82% of

the activity is lost when all four sites are mutated. The most dramatic effects of single mutations are seen when the mp5 (56% reduction) or F3/NF1 (52% reduction) binding sites are mutated. There is also a contribution of the F12 site to this enhancer since this mutation causes a 43% drop in activity. In 3T3 cells (Figure 3C), the most dramatic effects are seen with the mp4, F3/NF1, and F12 mutations that have reductions of 41%, 39% and 33% respectively. The mp5 mutation has the least effect in 3T3 cells (16% reduction) which is in contrast to its marked effect in 34i cells (56% reduction). In addition, mp4 has a greater effect in 3T3 cells (41% reduction) than in 34i cells (21% reduction). In summary, the Ban2 enhancer requires mp5, F3/NF1 and F12 in 34i mammary cells and mp4, F3/NF1 and F12 in 3T3 fibroblasts for optimal activity. The results of this mutation series prompted us to analyze the DNA-binding factors which interact with these regions in both mammary (34i) and non-mammary (3T3) cells.

Four DNA-binding proteins interact with sequences in the Ban2 enhancer.

DNA transfection experiments showed that at least four elements are required for optimal activity of the Ban2 enhancer in two different cell types. We employed mobility shift assays to determine if there are sequence-specific DNA-binding proteins associated with each of these elements. We first tested whether the proteins that have been reported to bind to the F3/NF1 and F12 elements are present in 34i and 3T3 nuclear cell extracts. In the following EMSA experiments, the sequences of the oligonucleotides are shown in materials and methods. Figure 4A shows a mobility shift assay using a labeled F3/NF1 probe (-1051 to -1019). We find that the F3/NF1 activity is present in both extracts and that the specific band competes with both unlabeled F3/NF1, and consensus NF1 (MMTV, -80 to -60) oligonucleotides (lanes 3,5,8 and 10) but not with an unrelated ATF binding site or an oligonucleotide containing a mutation in the consensus NF1 site, NF1m, (lanes

4,6,9 and 11). This data is in agreement the hypothesis of Cato and colleagues that a protein in the NF1/CTF family of transcription factors binds to the F3/NF1 element which contains an NF1 half-site (12). Figure 4B shows that the activity designated F12 is also present in 34i and 3T3 cells. The F12 (-1010 to -980) binding activity is competed by unlabeled F12 and F2 (lanes 3,4,9 and 10) oligonucleotides and is not competed by oligonucleotides containing ATF, NF1 or mp5 binding sites (lanes 5,6,7,11,12 and 13). The F2 element (-905 to -870) was also reported by Cato and colleagues to contain an F12 DNA-binding site (12). In addition to F3/NF1 and F12, we have identified an activity binding to the mp4 DNA sequence (-1075 to -1055) in both 34i and 3T3 cells (Figure 4C). The mp4 DNA-binding activity is specific because it is competed by an unlabeled mp4 oligonucleotide (lanes 3 and 6). The mp4m mutation targets a cis-acting sequence (ACAAAG) which is present in the murine WAP promoter and other mammary-specific promoters and has been shown to be important for mammary-specific expression of the WAP gene (22). Since the mp4m mutation partially competes the specific band (lanes 4 and 7), we hypothesize that either the mutation still allows for a weak mp4 interaction or that the mp4 protein is not the same protein that binds to the ACAAAG consensus sequence found in the WAP promoter.

The DNA-binding activity that we have designated mp5 is present in all cell types analyzed except for the liver carcinoma cell line HepG2. Figure 5 shows that there is a specific shifted complex present in the mammary cell lines T47D, MCF7, 34i and the non-mammary lines HeLa and 3T3 using mp5 as a probe. This band is competed by unlabeled wild type mp5 (lanes 3,6,9,16, and 19) but not an oligonucleotide containing a mutation in the mp5 consensus AP-2 site (lanes 4,7,10,17, and 20). The HepG2 extract is competent for EMSA studies because the mp4, F3/NF1 and F12 DNA-binding activities are all present in HepG2 cells (data

not shown).

The mp5 probe (-1044 to -1004) partially overlaps the F3/NF1 probe (-1051 to -1019) and contains an NF1 half-site (TGGCA) at its 5' end. Therefore, we designed an experiment to test whether the upper band (shaded arrow in Figures 2B and 5) represents an NF1 DNA-binding activity in 34i cells. In Figure 6, we used a ^{32}P-labeled mp5 probe and competed with either unlabeled NF1, NF1m, ATF or Met, an oligonucleotide containing a consensus AP-2 site from the human metallothionein IIa promoter (15). The upper band (shaded arrow) is competed by mp5, mp5m or NF1 (lanes 2, 3 and 4) but not by NF1m or ATF (lanes 5 and 6). This shows that the upper band contains an NF1-like DNA-binding activity. The Met oligonucleotide competes the upper and lower shift indicating that AP-2 may be required for the NF1-like binding activity and that there may be cooperative binding between mp5/AP-2 and NF1. Alternatively, the upper band may represent an NF1 homo or hetero-dimer binding exclusively to the probe, although the MMTV (-81 to -59) NF1 mobility shift has a faster mobility than the mp5 shift (data not shown). The faint broad band in the HepG2 mp5 shift with no competitor competes with both wild type mp5 and mp5m which both contain the NF1 half-site (Figure 5; compare lanes 11,12 and 13). Additional studies show that this activity is competed with NF1 but not NF1m indicating that this band most likely represents an NF1 homodimer DNA-binding activity (data not shown).

HepG2 is the only cell line tested that does not contain the mp5 binding activity and it has been previously shown to be lacking in AP-2 (15). This data in combination with the fact that there is a consensus binding site for AP-2 in the mp5 element prompted us to investigate whether mp5 is in fact AP-2.

The mp5 DNA-binding activity is related to AP-2.

If the mp5 protein is antigenically related to AP-2, then the EMSA mp5 band should be supershifted by antibodies to AP-2. Ab53 and Ab57 are two affinity

purified anti-AP-2 polyclonal antisera made against peptides from different regions of the human AP-2 protein. We tested each anti-AP-2 antibody in two human (T47D and HeLa) and two murine (34i and 3T3) cell extracts and the results are shown in Figure 7. As a positive control, we show that *in vitro* translated AP-2 (IVT-AP-2) is quantitatively supershifted by Ab53 (lanes 13 and 14). There are no non-specific bands present when either Ab53 or Ab57 are added to probe in the absence of extract or when an unrelated anti-glutathione-S-Transferase affinity purified antibody is added to the extract (data not shown). In each cell extract, both Ab53 (lanes 2,5,8 and 11) and Ab57 (lanes 3,6,9 and 12) are capable of supershifting the mp5 DNA-binding activity.

This experiment also shows, importantly, that IVT-AP-2 binds to the mp5/AP-2 element present in our mp5 probe and that IVT-AP-2 co-migrates with the mp5 DNA-binding activity in our cell extracts (compare lane 13 to lanes 1, 4, 7 and 10). In addition, we have performed EMSA protease clipping assays with the mp5 probe which show that the limit digest of T47D, HeLa, 34i and 3T3 cell extracts co-migrate with IVT-AP-2 (data not shown). The amino acid sequences of the human and murine AP-2 proteins are 99% identical so it is predicted that the protein in murine extracts would have a similar protease digestion profile (23).

In summary, the above experiments show that the mp5 activity (now designated mp5/AP-2) is either AP-2 or an antigenetically related family member. To test for functional involvement of the AP-2 protein in the activity of the Ban2 enhancer, we conducted AP-2 expression experiments in a cell line that lacks endogenous AP-2.

AP-2 is required for the activity of the Ban2 enhancer in HepG2 cells.

It has been previously shown that the cell line HepG2 lacks endogenous AP-2 activity (15). We demonstrate here that the Ban2 enhancer has no stimulatory activity in HepG2 cells (Figure 1), and that the mp5/AP-2 DNA-binding activity is

absent in nuclear extracts from these cells (Figure 5, Lanes 11,12,13). The mp4, F3/NF1, and F12 DNA-binding activities are all present in HepG2 cells (data not shown) indicating that mp5/AP-2 may be critical for the activity of this enhancer . We co-transfected a eukaryotic expression vector for AP-2 (pRSVAP-2) with the pBan2TK81 construct into HepG2 cells. In this assay, we tested for the activity of the Ban2 enhancer in the presence or absence of exogenous AP-2. As a positive control for the efficiency of expression of the pRSVAP-2 vector, we utilized a control vector, pAP-2BCAT, containing 3 AP-2 DNA-binding sites cloned directly upstream of the E1b TATA box of pBCAT (15). In this case, the pAP-2BCAT vector was stimulated 5-fold in the presence of co-transfected pRSVAP-2, indicating that the expressed AP-2 protein is functional in HepG2 cells. The RSV promoter vector alone has no effect on the activity of pBan2TK81or pTK81 (data not shown).

Figure 8 shows the results of DNA transfection experiments in HepG2 cells. The data is presented as the ratio of activity for cells co-transfected with Ban2 reporters and pRSVAP-2, relative to the activity observed with cells that had only Ban2 reporters (AP-2+/AP-2-). The pBan2TK81 vector was stimulated an average of 6.5-fold in the presence of pRSVAP-2. These results demonstrate that AP-2 is required for the activity of this enhancer in HepG2 cells. In addition to the wild type enhancer, we tested the mutation series that was used in the initial characterization of the enhancer (Figure 3A). The mp4 and F12 mutations have no significant effect on the ability of AP-2 to stimulate the enhancer. In contrast, the enhancers containing the mp5 mutation alone (mp5m), or all four sites mutated [(4X)m], are not capable of responding to AP-2. This shows that the mp5/AP-2 sequence element that we have mutated in both of these constructs is the target for exogenous AP-2 activity. We note that the F3 mutation is compromised by 30% in its ability to respond to AP-2. This is further evidence that there may be a functional interaction between F3/NF1 and AP-2.

Discussion

Transcriptional regulation of MMTV is complex; the LTR has been shown to contain multiple regulatory elements, both positive-acting and repressive (24). We previously reported that the 5' end of the LTR contained a functional enhancer that was selectively active in cells of mammary origin, and presented evidence for one factor, mp4, that binds to this element (11). Two other groups, Yanagawa et al. (13) and Cato and colleagues (12) have also documented the tissue-specific activation potential of sequences in this region, and have reported several proteins binding to these sequences.

We have now characterized in some detail an element located at the extreme 5' end of the LTR, the Ban2 restriction fragment (-1075 to -978), that functions as a cell-selective enhancer in cultured cells. We show that four DNA-binding proteins interact with this 100 bp region and that one of the proteins, mp5, is either AP-2 or a closely related protein. Our results also confirm the findings of Cato and colleagues that two factors, F3/NF1 and F12, interact with this enhancer. In addition, we provide functional and mobility shift evidence for the mp4 factor (11).

The most significant reduction in transcriptional activity of the Ban2 enhancer in mammary cells results from mutation of the mp5/AP-2 site. This mutation fails to completely inhibit Ban2 enhancer activity in 34i cells, however. Thus, mp5/AP-2 is not the only element required; binding sites for F3/NF1 and F12 are also required for enhancer activity in 34i cells. In 3T3 cells, the mp5/AP-2 mutation has only a 16% effect even though the AP-2 protein is expressed in this cell line. None of the mutations that we analyzed completely eliminate the activity of this enhancer when tested alone, indicating that there is functional redundancy of these elements, or that there is an additional required activity that has not been identified. We hypothesize that AP-2, F3/NF1 and F12 regulate this enhancer in a combinatorial fashion. In fact, *in vivo* 5' exonuclease assays show that mp4, F3/NF1, mp5/AP-2 and F12 are

each bound to their respective DNA elements with boundaries consistent with *in vitro* derived DNaseI footprints (25). Since the binding sites for mp5/AP-2 and F3/NF-1 are adjacent to each other it is conceivable that the two activities may be interacting; it will be interesting to determine whether they bind in combination or in a mutually exclusive manner *in vitro* and *in vivo*. In either case, it is possible that small fluctuations in the concentration of either F3/NF1 or mp5/AP-2 would have significant effects on the activity of this enhancer.

AP-2 has been shown in several systems to interact with other transcription factors which bind to overlapping or juxtaposed cis-acting sequences. First, AP-2 binds to a promoter element in the human growth hormone gene in a mutually exclusive manner with NF1 (26). The DNA-binding domains of NF1 and AP-2 share no homology, but it may be significant that they both contain a proline-rich activation domain. Second, AP-2 binds to overlapping sequences with a factor called AP-3 on the 'core' sequence of the SV40 enhancer and when AP-2 is bound to the element AP-3 is excluded (27). Third, AP-2 has been shown to bind to an a enhancer element in the H-2κ^b gene promoter which is activated by TNF-α and in TNF-α treated cells AP-2 is replaced by an NFκB-like binding activity (28).

AP-2 activity is also regulated by several factors that inhibit its binding to DNA. It was recently shown that a second splice form of the AP-2 protein exists in several cell types. This protein, called AP-2B, results from differential exon usage and is identical to AP-2 at the N-terminus but is lacking the DNA-binding domain (29). Buettner and colleagues show by EMSA that AP-2B inhibits the ability of AP-2 to bind to DNA and by transfection analysis that AP-2B inhibits the functional activity of AP-2. They also show that AP-2 and AP-2B do not bind to each other directly but require an undefined factor that is present in rabbit reticulocyte lysate or mammalian cell extracts but not in bacterial extracts. This raises the possibility that differences in the levels of AP-2, AP-2B and a potential bridging factor will affect

gene regulation in different cell types. AP-2 activity has also been shown to be inhibited by SV40 T antigen and HTLV-1 Tax which prevent the binding of AP-2 to DNA in DNaseI footprinting and mobility shift assays, respectively (30,31).

What is the mechanism by which the Ban2 enhancer is preferentially active in mammary cells? The lack of activity in HepG2 cells is straightforwad; AP-2 is simply not present in these cells. The data of Ross and collaborators (10) suggests that a fragment encompassing the Ban2 region of the LTR manifests very pronounced tissue-targeting activity in transgenic animals, even when attached to a heterologous promoter. The enhancer is not quiescent in all non-mammary cells in culture, however, as shown here for 3T3 cells. The enhancer is also modestly active in HeLa cells (32). Incomplete restriction of cell type specificity could have several explanations. Cells grown on plastic substrata in culture lose many of the features of primary cells. Assembly of an active multifactor complex on the enhancer in inappropriate cell types could result from aberrant modification of one or more of the factors, or loss of secondary factors blocking the binding of one of the activators. As noted above, an AP-2 variant that blocks normal AP-2 activity has already been described.

Alternatively, the activation potential of the Ban2 enhancer may be modulated in some cells by other repressive elements in the LTR. It is becoming increasingly clear that both positive and negative elements are responsible for selective expression of MMTV in the mammary gland. Several negative regulatory elements have been described. One DNA element (-428 to -364) has been characterized both in transfection experiments (33,34) and by analysis of proviral sequences that are deleted in T-cell lymphomas (4). In agreement with these studies, analysis of the LTR in transgenic mice shows that there is a negative regulatory element which maps between -665 and -165 (35). A second negative element (-156 to -162) has been defined between the two distal HRE's and has been shown to bind a nuclear

factor (36).

Thus, the ability of the Ban2 enhancer to activate transcription may be conditioned by repressive elements between the enhancer and the promoter. In this context, it is important to note that the orientation of proviral elements activating the *wnt*-2 proto-oncogene is invariably antiparallel with respect to the activated promoter, even with proviruses inserted at large distances from the oncogene (7,8). Thus, the Ban2 enhancer is always placed at the end of the LTR in the direction of the target promoter. The significance of such directional positioning of elements over large distances is unclear. The Ban2 element, however, is clearly a candidate for the major activator of cellular *wnt* genes during proviral insertion and tumorigenesis.

In summary, we have now defined an activating element at the 5' end of the MMTV LTR that provides one component of the cell specificity observed for expression from this promoter. The involvement of AP-2, or a closely related protein, has been established as one of the primary factors acting at this locus. Further efforts will now be directed at a complete delineation of the AP-2 forms present in mammary cells, post-translational modifications which may be specific to these cells, and the mechanisms involved in assembly of the complex multifactorial protein complex binding to this locus.

Future experiments will focus on the proteins which bind two of the elements that we have shown to be the most critical for the activity of the Ban2 enhancer. These proteins AP-2 and an NF1-like factor each belong to a family of related proteins. The AP2 family contains at least three members called AP2-α, AP2-β and AP2-γ. The AP2-γ protein is of particular interest because it was originally cloned as OB2, a protein which binds to and has been shown to regulate the c-erbB2 gene promoter (37). c-erbB2 is a protein that is overexpressed by both gene amplification and promoter activation in at least 50% of human breast carcinomas.

AP2-α/OB2 is upregulated in the human breast cancer cell lines in which the c-erbB2 gene is overexpressed. Therefore AP2-α may be important for the development of human breast cancer. We plan to investigate which family member of AP2 regulates the MMTV mammary-specific element. The second protein, NF1, is a member of a large family of proteins that bind to the CAAT box sequence. We have initiated experiments to purify the factor which binds to the MMTV sequences to determine whether it is a novel member of this family.

In addition, we have initiated several current lines of investigation that continue to address my statement of work. In view of the fact that the MMTV LTR is most highly expressed in the mammary glands of mice and is upregulated during lactation, we have started using a tissue culture system that more closely models the mammary gland during development. This system was originally developed in the laboratory of Dr. Mina Bissell (38). They are using a mammary murine epithelial cell strain (CID-9) that maintains the ability to differentiate on extracellular matrix (ECM) to form a three dimensional alveolar-like structure. In the presence of lactogenic hormones and ECM, CID-9 cells secrete milk proteins such as beta-casein into a lumen. We have exploited this tissue culture system and have preliminary data to show that the MMTV LTR is upregulated when these cells are differentiated. We are currently investigating which DNA elements in the LTR are required for this secondary level of gene regulation that may require the remodeling of the nuclear matrix as is seen when CID-9 cells are grown on ECM. We are also in the process of making constructs for the transgenic mouse studies that will be based on our knowledge of the function of this enhancer in tissue culture cells.

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Figure Legends

Figure 1

DNA transfection analysis of the Ban2 enhancer. A) Diagram of the MMTV LTR showing the U3 and U5 regions and the location of fragments that have been shown to be important for tissue specificity. The line labeled Transgene represents a fragment that directs the expression of a heterologous promoter to mammary gland in transgenic mice (10). The fragments Ban2 and Pst-Hinf1 were shown to be mammary-specific enhancers when driving the MMTV minimal promoter and in the case of Ban2 a heterologous promoter (11,13).

B) Activity of the Ban2 enhancer in 34i, 3T3, and HepG2 cells. pTK81 (Vector) is standardized to 1 therefore the y-axis represents the ratio of pBan2TK81/pTK81 (Ban2) for each cell line. The bar graph shows the mean and standard error for 5 DNA transfection experiments for 34i and 3T3 and 4 experiments for HepG2 done in duplicate on separate days. The y-axis represents luciferase/β-galactosidase activity.

Figure 2

EMSA using the mp5 sequence as a probe to delineate the mp5 DNA-binding site.

A) The coding strand of double-stranded oligonucleotides used for this experiment. The oligonucleotide designated mp5 contains sequences from the C3H MMTV LTR. Coordinates are labeled relative to the start site of transcription. The competitors are identical in sequence to the mp5 probe except for the indicated mutations. The dashes indicate the inferred binding site based on the mobility shift data in 2B. The consensus AP-2 DNA-binding sequence is shown as previously determined by Williams et al (15).

B) EMSA to determine which sequences are required for mp5 DNA-binding

activity in 34i cells. mp5 is the ^{32}P-labeled probe and the competitors (shown in A) are used at 100x probe concentration. Lane 1 has no competitor and lane 13 has an unrelated ATF binding site (see Materials and Methods). The mutation in the wapm oligonucleotide targets a sequence shown to be important for WAP gene promoter activity (22). The open arrow denotes the specific mp5 band and the shaded band denotes a second binding activity that is discussed in Figure 6.

Figure 3

The activity of pBan2TK81 and the mutation series in 34i mammary carcinoma cells and 3T3 fibroblasts. A) Diagram of the Ban2 fragment and mutated fragments which were cloned into pTK81. The MMTV LTR coordinates are numbered relative to the start site of transcription. The diagram shows 4 DNA-binding proteins that interact with this element and their predicted binding sites based on DNaseI footprinting (11,12,20,25). The sequences in the mutated fragments are identical to the wild-type except for the indicated changes. For mp4m, F3m, F12m, and mp5m the wild type sequence is shown below the line and the mutation shown above and for mp4+5m and (4x)m only the mutated bases are shown.

B and C) The bar graphs represent the mean and standard error from 5 independent DNA transfections for mp4m, F3m, F12m and 4+5m and the mean of 2 experiments for mp5m and (4x)m done in duplicate on separate days. The y-axis represents luciferase/β-galactosidase activity and the values for each mutated plasmid are expressed as a percentage of wild type Ban2 activity. The following values determined the mean percentage of wild type for mp5m and (4x)m: 34i cells, mp5m (43,44) , (4x)m (21,15); 3T3 cells, mp5m(80,87) and (4x)m (28,42). The dotted line highlights the percentage of wild type activity that is contributed by pTK81 vector alone.

Figure 4

EMSA using F3/NF1, F12 and mp4 as probes in 34i and 3T3 cell extracts. The open arrows indicate the specific band for each gel shift. The sequences of both probes and competing oligonucleotides are shown in materials and methods and competitors are used at 100x probe concentration. A) EMSA using an F3 ^{32}P-labeled probe in 34i and 3T3 nuclear cell extracts. B) EMSA using an F12 ^{32}P-labeled probe in 34i and 3T3 nuclear cell extracts. C) EMSA using a mp4 ^{32}P-labeled probe in 34i and 3T3 nuclear cell extracts.

Figure 5

EMSA showing the mp5 DNA-binding activity in both human and murine cell lines. Mobility shifts are shown for human T47D (lanes 2,3,4), MCF-7 (lanes 5,6,7), HeLa (lanes 8,9,10), HepG2 (lanes 11,12,13) and murine 34i (lanes 15,16,17) and 3T3 (lanes 18,19,20) cell lines. The mp5 ^{32}P-labeled probe and the mp5m competitor (used at 100x) are identical to the oligonucleotides described in Figure 1. The open arrow indicates the specific mp5 shift and the shaded arrow indicates a second activity which is discussed in Figure 6.

Figure 6

EMSA in 34i nuclear cell extracts showing that the upper mp5 shift represents and NF1-like DNA-binding activity. The mp5 sequence is used as a ^{32}P-labeled probe and the sequences of competitors (used at 100x) are shown in materials and methods. The open arrow indicates the mp5 consensus band and the shaded arrow indicates an NF1-like activity.

Figure 7

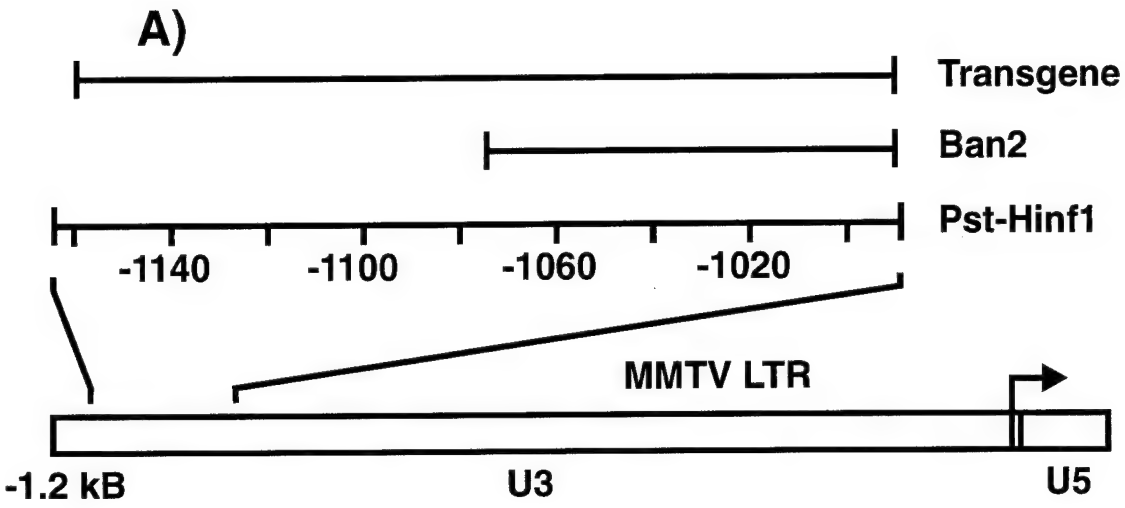
EMSA showing that the mp5 activity is supershifted by anti-AP-2 antibodies.

mp5 is the ^{32}P-labeled probe and the cell extracts are T47D (lanes 1,2,3), HeLa (lanes 4,5,6), 34i (lanes 7,8,9),and 3T3 (lanes 10,11,12). Lanes 13 and 14 are the shifts obtained with 1µl IVT-AP-2 protein. The mp5/AP-2 specific band is indicated by an open arrow and the band which represents the supershift for Ab53 (lanes 2,5,8,11 and 14) and Ab57 (lanes 3,6,9, and 12) is indicated by a shaded arrow. In order to eliminate the upper EMSA band, which represents an NF1 DNA-binding activity (see figure 6) that co-migrates with the AP-2-Ab complex, a 100-fold molar excess of unlabeled NF1 oligonucleotide relative to probe was added to each reaction. The free mp5 ^{32}P-labeled probe is run off the gel in order to resolve the protein-Ab-DNA complex.

Figure 8

DNA transfection experiment showing that AP-2 is required for Ban2 enhancer activity in HepG2 cells. The bar graph shows the activity of the Ban2 wild-type, mp4, F3m, mp5m, (4X)m, and the pTK81 control represented as a ratio of cells co-transfected with pRSVAP-2 to cells with reporter alone for each construct. This data represents the mean and standard error for five independent DNA transfection experiments for WT, mp4m, mp5m and (4x)m and 7 experiments for F3m and F12m done in duplicate on separate days. The activity of Ban2-wt (see Figure 1) and each of the mutant constructs in the absence of pRSVAP-2 is similar to pTK81.

Figure 1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-1060
-1020
MMTV LTR
-1.2 kB
U3
U5

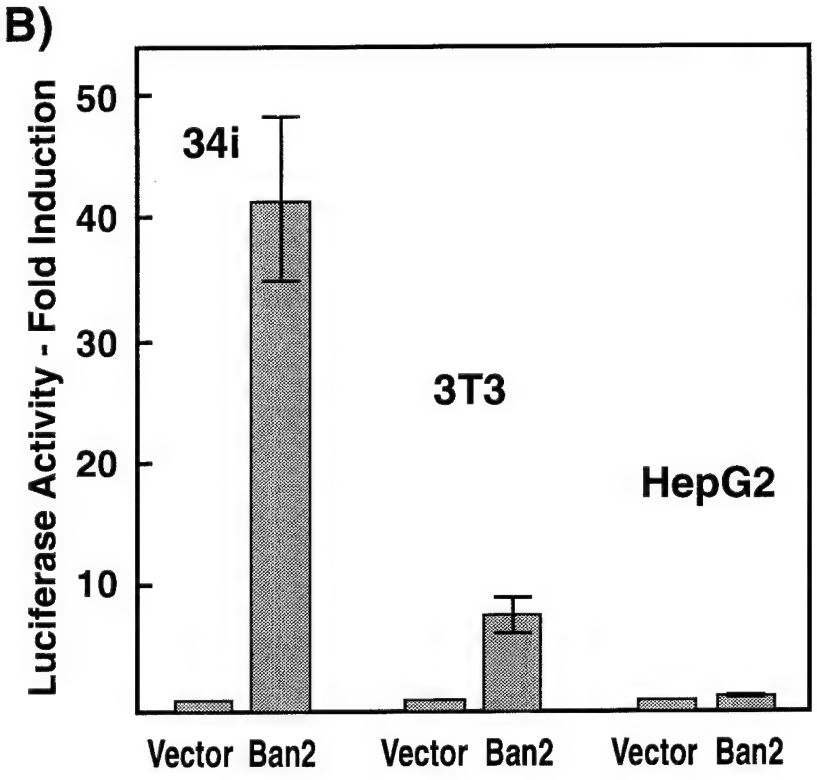
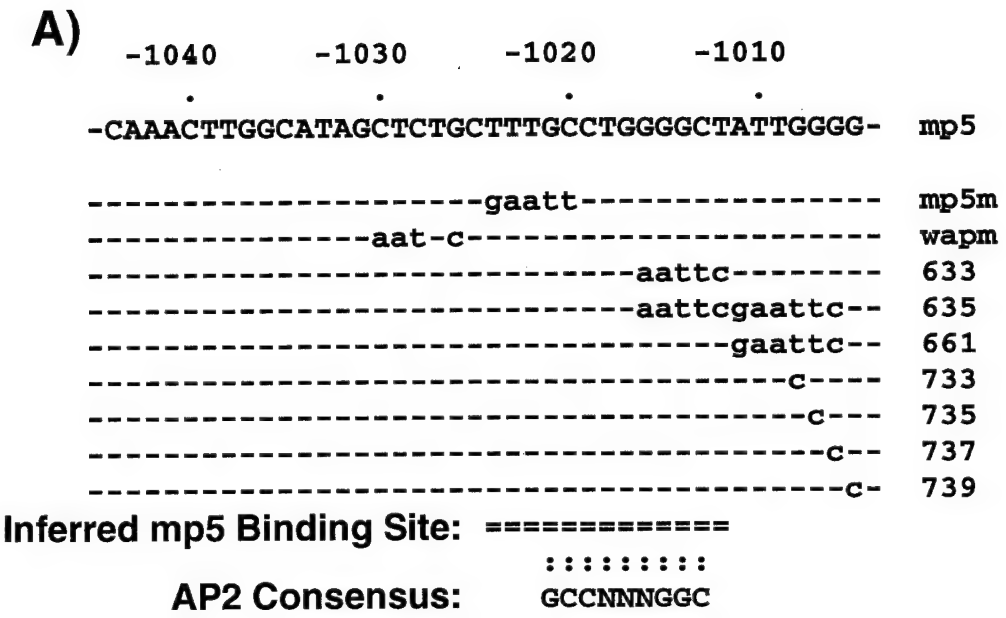

B)
Luciferase Activity - Fold Induction
50
40
30
20
10
34i
3T3
HepG2
Vector Ban2
Vector Ban2
Vector Ban2

Figure 2A


A)
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-1020
-1010
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----------gaatt---------- mp5m
----------aat-c---------- wapm
----------aattc--------- 633
----------aattcgaattc-- 635
----------gaattc-- 661
----------c---- 733
----------c--- 735
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Inferred mp5 Binding Site: ==========
:::::::::
AP2 Consensus: GCCNNNGGC

Fugure 2B

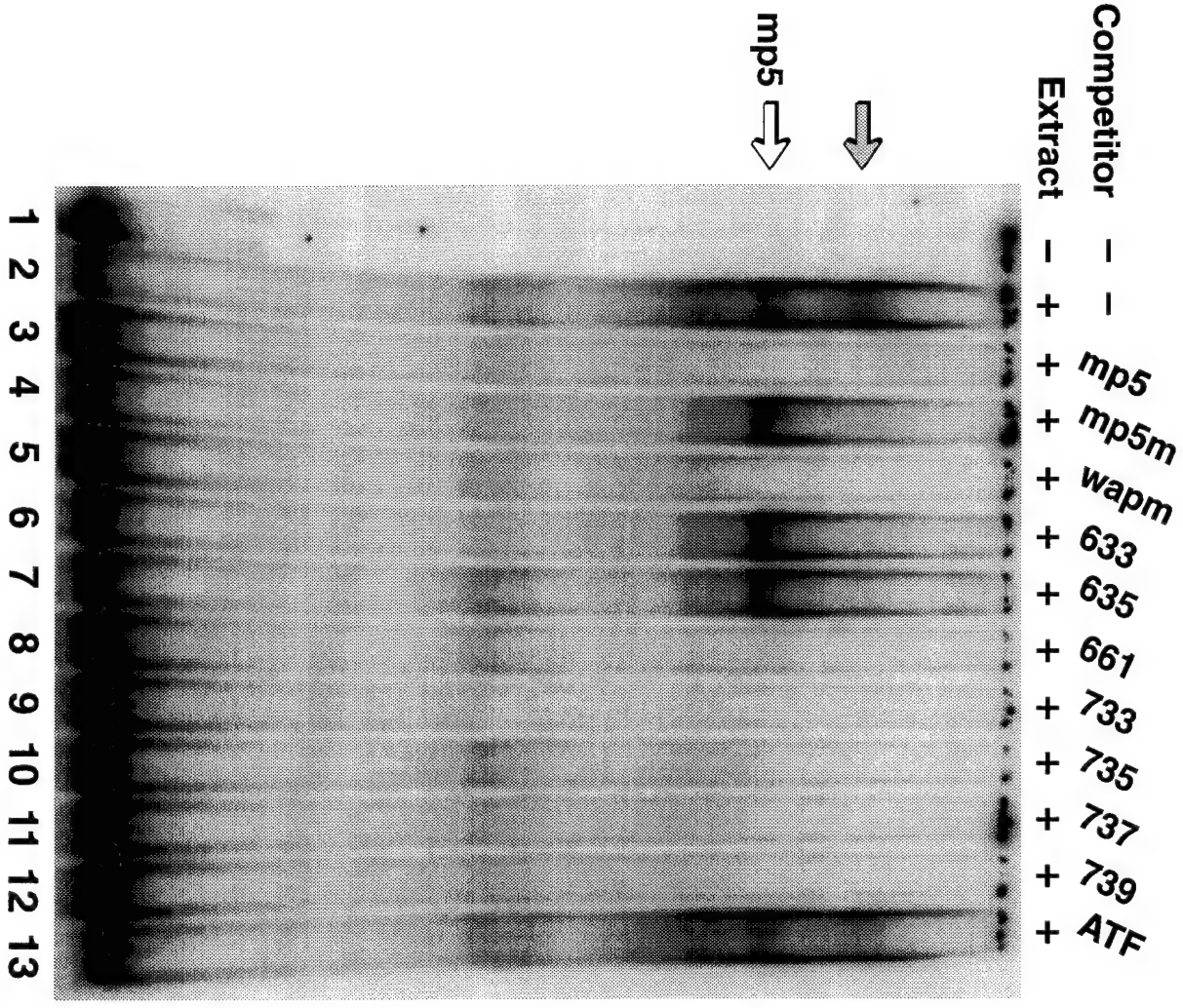
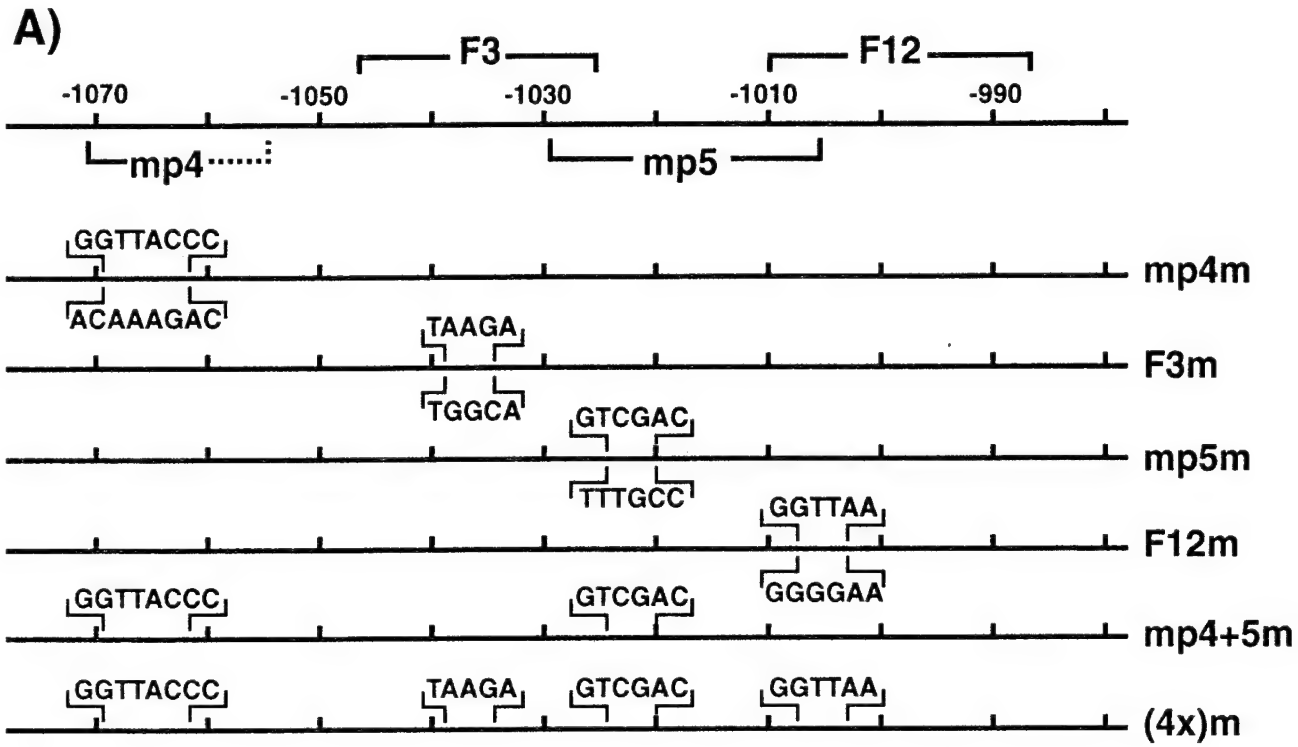

Competitor
Extract
– –
+ –
+ mp5
+ mp5m
+ wapm
+ 633
+ 635
+ 661
+ 733
+ 735
+ 737
+ 739
+ ATF
mp5
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3A


A)
F3
F12
-1070
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-1030
-1010
-990
mp4
mp5
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TGGCA
F3m
GTCGAC
TTTGCC
mp5m
GGTTAA
GGGGAA
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GGTTAA
(4x)m

Figure 3 B and C

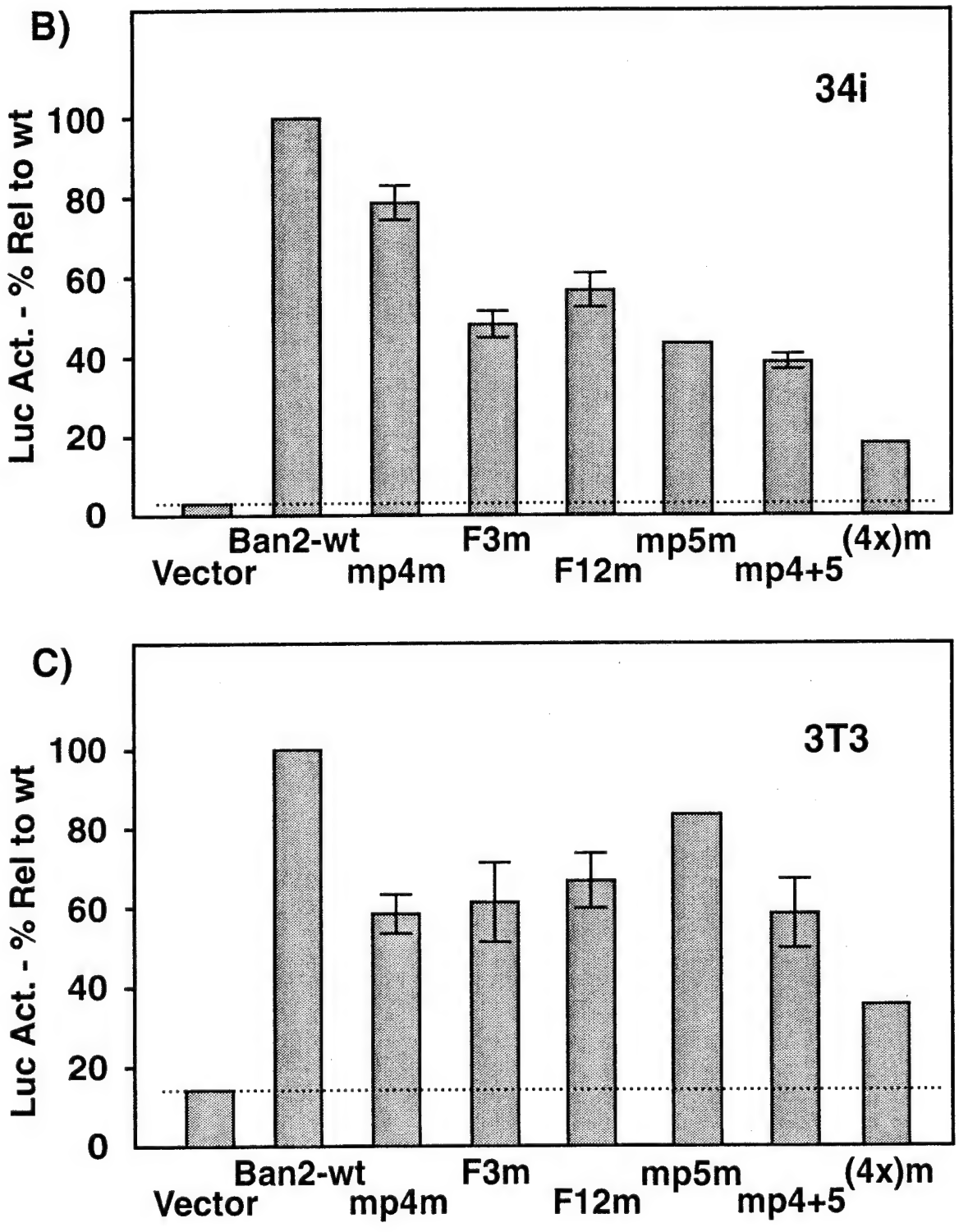


Figure 4

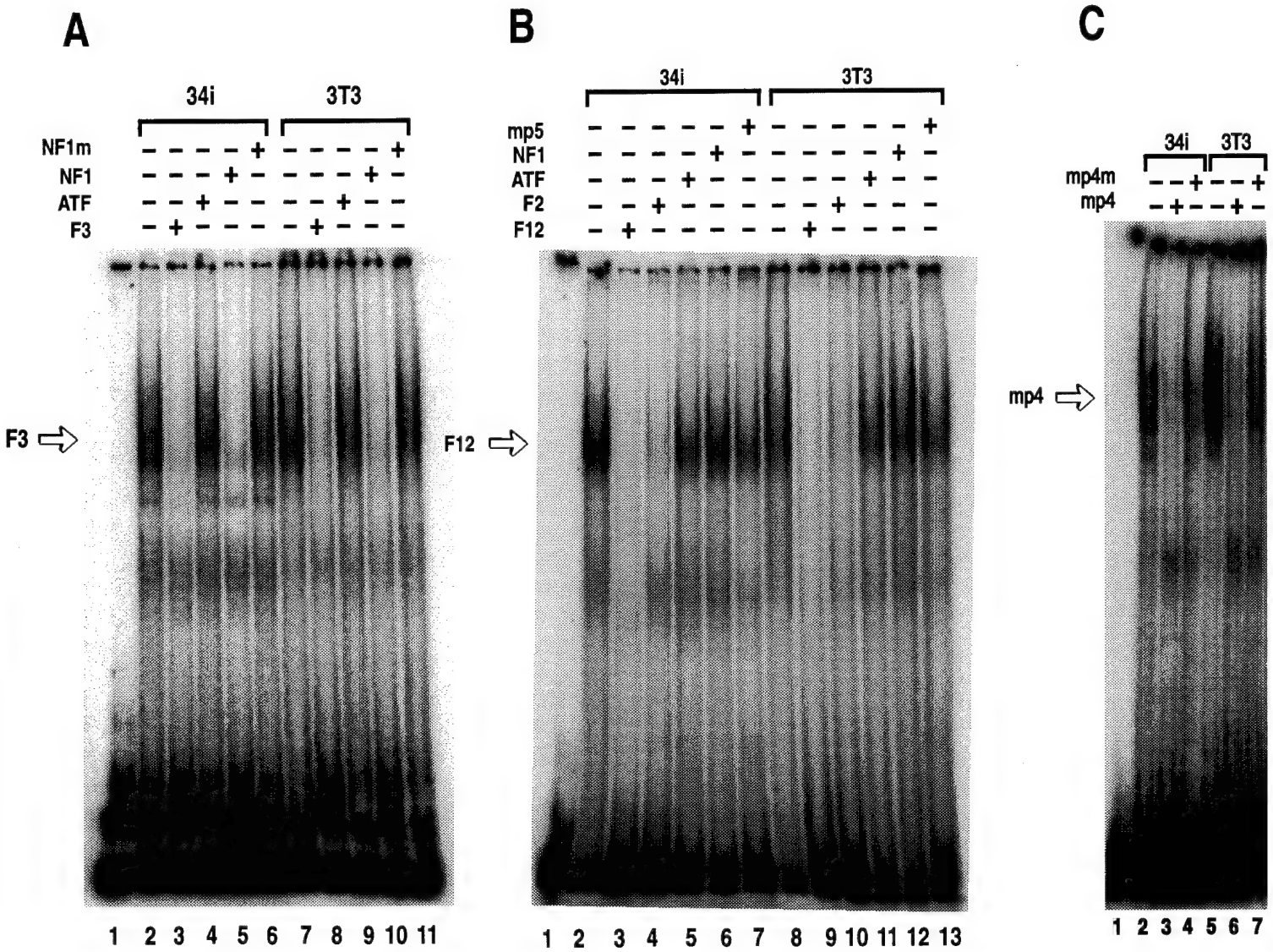

A
34i
3T3
NF1m
NF1
ATF
F3
F3
1 2 3 4 5 6 7 8 9 10 11
B
34i
3T3
mp5
NF1
ATF
F2
F12
F12
1 2 3 4 5 6 7 8 9 10 11 12 13
C
34i
3T3
mp4m
mp4
mp4
1 2 3 4 5 6 7

Figure 5

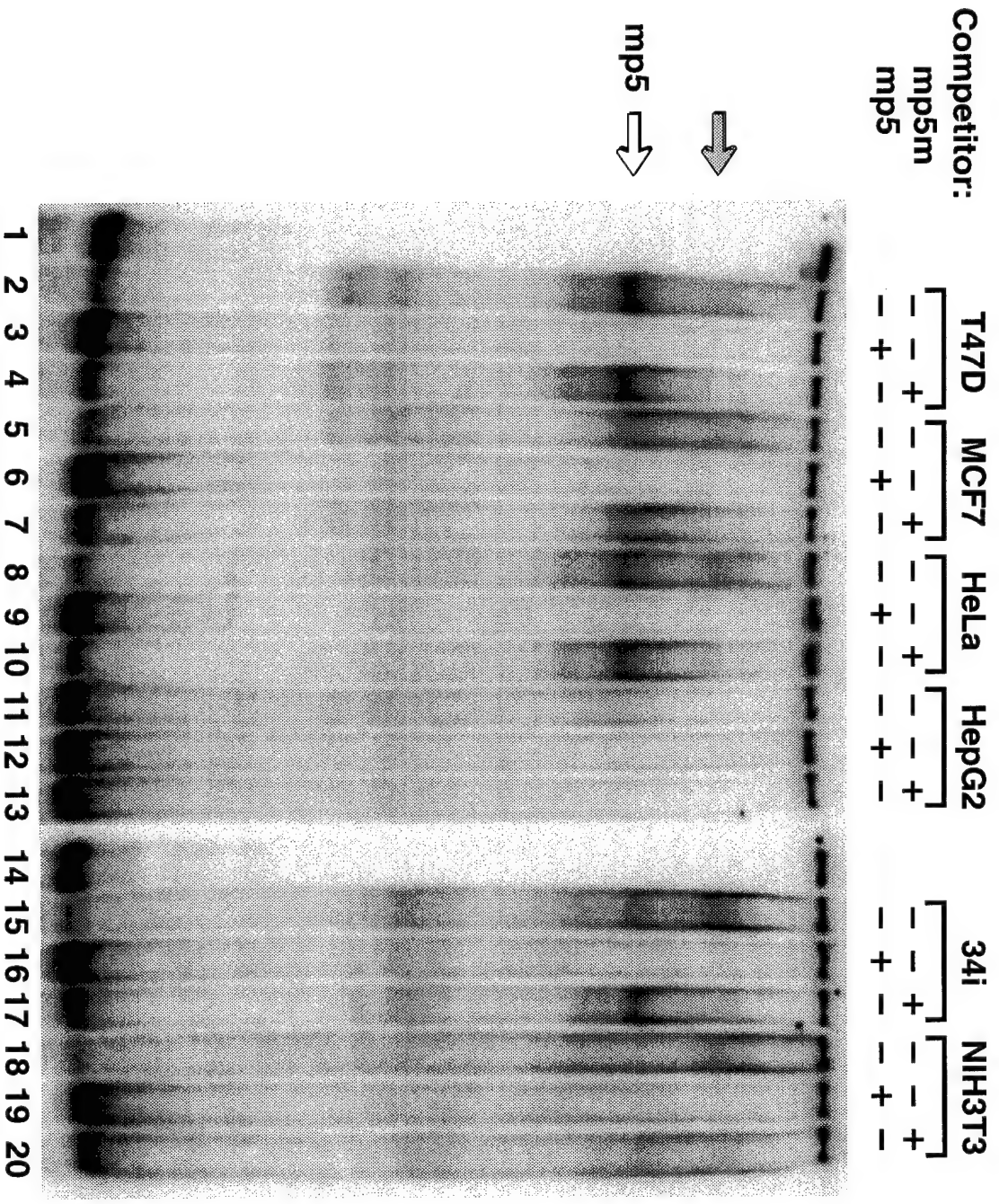

Competitor:
mp5m
mp5
T47D
MCF7
HeLa
HepG2
3Ti
NIH3T3
mp5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 6

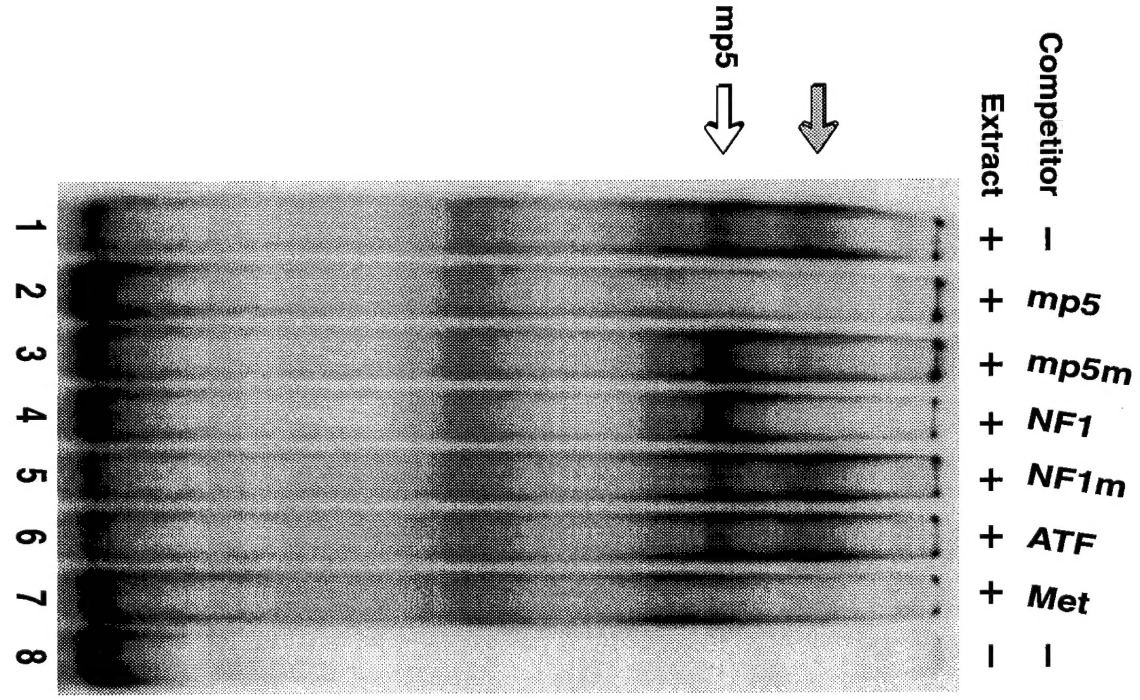


Figure 7

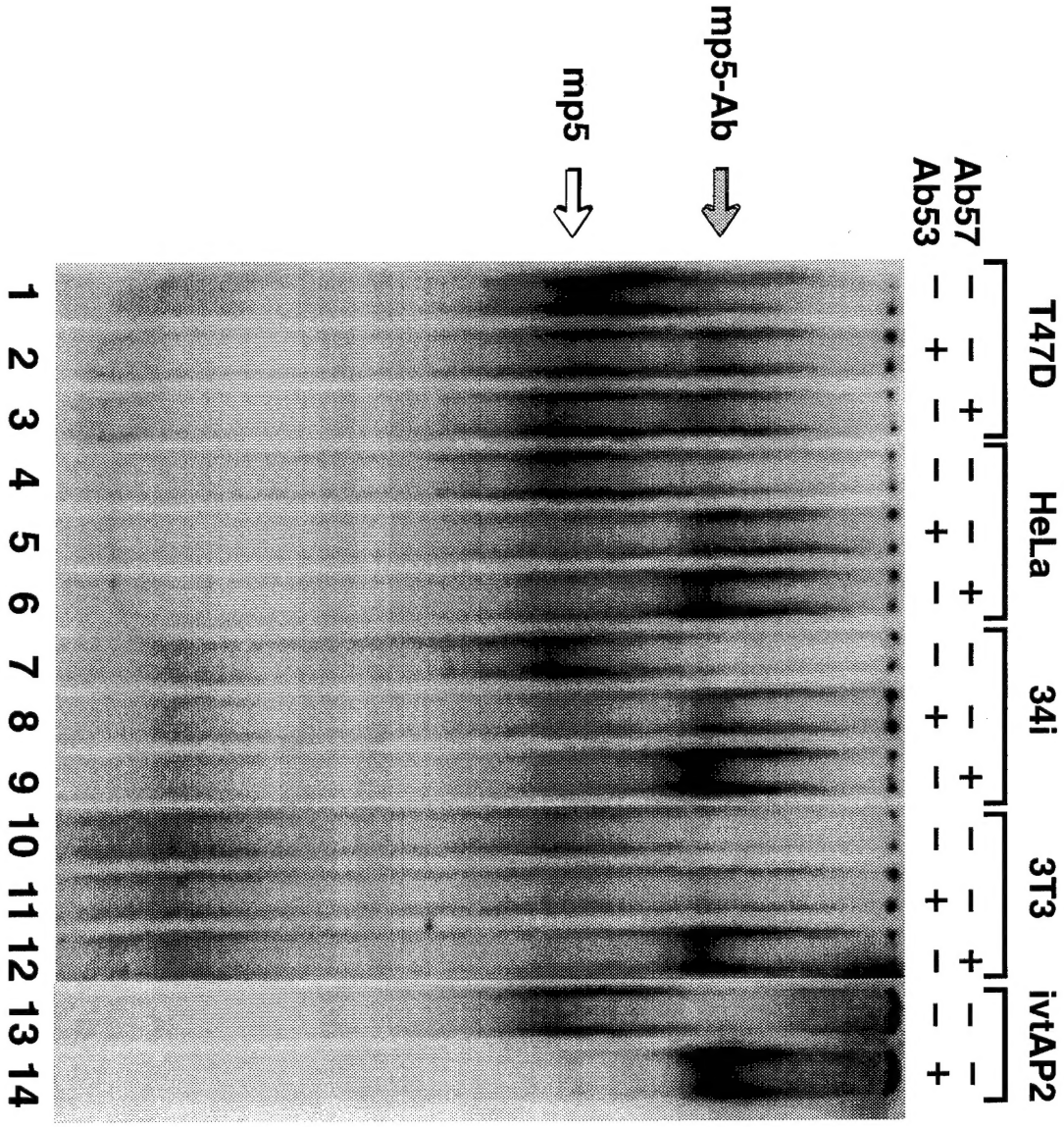
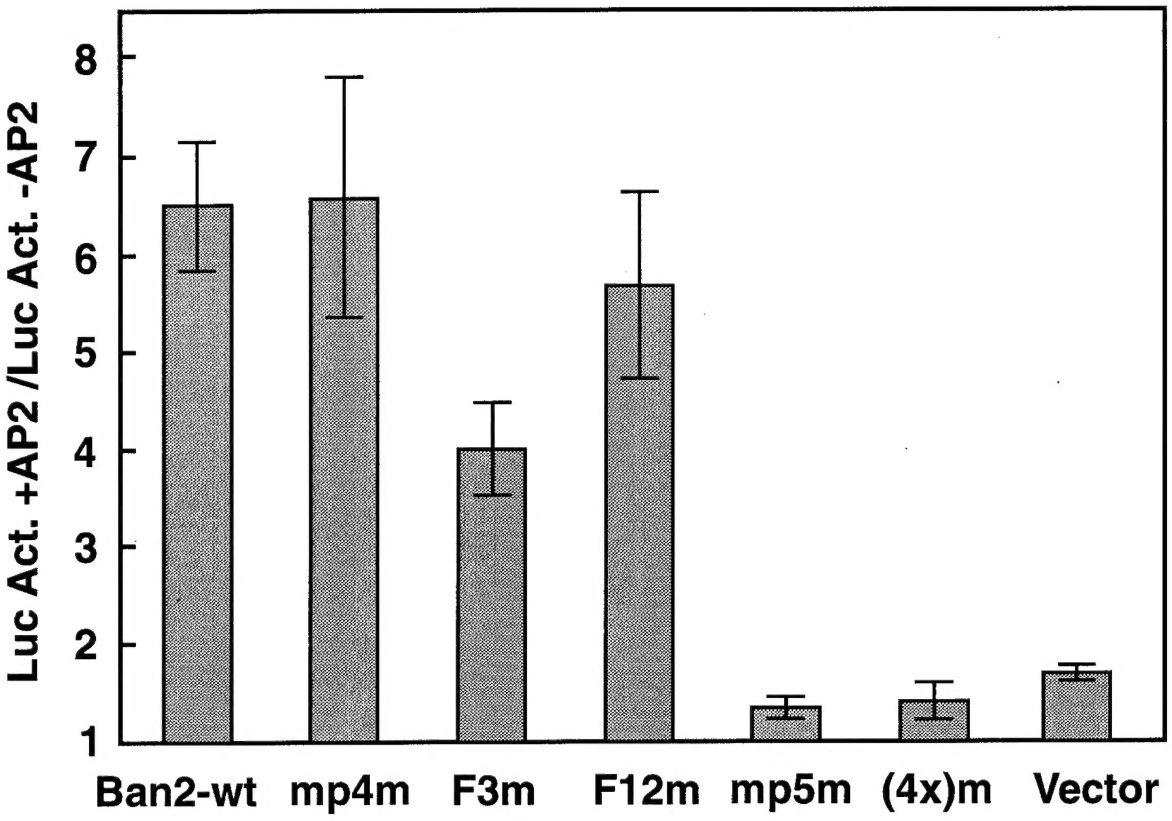

T47D
HeLa
34i
3T3
ivtAP2
Ab57
Ab53
mp5-Ab
mp5
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 8


Luc Act. +AP2 /Luc Act. -AP2
8
7
6
5
4
3
2
1
Ban2-wt
mp4m
F3m
F12m
mp5m
(4x)m
Vector

Collaborators

This work was done in collaboration with Sam John and Gordon Hager.

Work in progress is being done with Connie Myers, Philip Kusk and Gordon Hager.